

# The Wnt signaling mediator tcf1 is required for expression of *foxd3* during *Xenopus* gastrulation

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**ABSTRACT** TCF1 belongs to the family of LEF1/TCF transcription factors that regulate gene expression downstream of Wnt/ $\beta$ -catenin signaling, which is crucial for embryonic development and is involved in adult stem cell regulation and tumor growth. In early *Xenopus* embryos, tcf1 plays an important role in mesoderm induction and patterning. *Foxd3* emerged as a potential tcf1 target gene in a microarray analysis of gastrula stage embryos. Because *foxd3* and tcf1 are coexpressed during gastrulation, we investigated whether *foxd3* is regulated by tcf1. By using morpholino-mediated knockdown, we show that during gastrulation *foxd3* expression is dependent on tcf1. By chromatin immunoprecipitation, we also demonstrate direct interaction of  $\beta$ -catenin/tcf complexes with the *foxd3* gene locus. Hence, our results indicate that tcf1 acts as an essential activator of *foxd3*, which is critical for dorsal mesoderm formation in early embryos.

**KEY WORDS:** *foxd3*, *lef1/tcf*, *tcf1*, *Xenopus*, *Wnt*

## Introduction

LEF1/TCF transcription factors perform essential functions in developing embryos and in the maintenance of stem cell compartments in adult tissues (Kléber and Sommer, 2004; Logan and Nusse, 2004). Most vertebrates have four *LEF/TCF* genes: *TCF1* (*TCF7*), *TCF3* (*TCF7L1*), *TCF4* (*TCF7L2*) and *LEF1*. Alternative splicing and/or promoter use in these genes can generate a variety of isoforms, which are involved in differential tissue-specific and stage-specific Wnt responses (Arce *et al.*, 2006). All these transcription factors bind DNA via their common, highly conserved HMG box and mediate Wnt signaling in the nucleus by recruiting  $\beta$ -catenin and co-activators to Wnt response elements in the target genes. In the absence of Wnt signaling, LEF/TCF factors can also be bound by groucho factors, mainly to repress target gene transcription (Brantjes *et al.*, 2001). The *Xenopus* embryo is one of the best understood vertebrate model systems for investigating

the role of Wnt signaling via *lef1/tcf* factors in dorsoventral axis determination (reviewed in (Hoppler and Kavanagh, 2007)). The early *Xenopus* embryo expresses three maternally inherited *lef1/tcf* family members: *tcf1*, *tcf3* and *tcf4* (Houston *et al.*, 2002; Molenaar *et al.*, 1996; Roël *et al.*, 2003). Zygotic expression of these genes and of *lef1* increases at the start of gastrulation (König *et al.*, 2000; Molenaar *et al.*, 1998; Roël *et al.*, 2003, 2009).

In early *Xenopus* embryogenesis, mesodermal cell fate is induced at the equator of the embryo (also called the marginal zone). During subsequent gastrulation movements, the mesoderm is further specified and moves into the embryo, whereby the ventrolateral zone of the pre-gastrulating embryo will give rise to the somite forming paraxial and ventral mesoderm, and the dorsal organizer zone will form the head mesoderm and notochord.

*Abbreviations used in this paper:* ChIP: chromatin immunoprecipitation; Dex: dexamethasone; MO: morpholino.

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At the time of gastrulation, *tcf1* mRNA is present at high levels in the animal cap and in most of the marginal zone (Roël *et al.*, 2003). Maternal loss-of-function experiments have shown that *tcf1* functions as an activator of dorsal genes, but can also repress ectopic expression of these genes on the ventrolateral side of the embryo (Standley *et al.*, 2006). Furthermore, zygotic knockdown experiments revealed that *tcf1* plays a role in mesoderm induction and, along with *lef1*, in subsequent patterning of the ventrolateral mesoderm (Liu *et al.*, 2005). These studies have shown that in the *Xenopus* embryo, *tcf1* acts cooperatively with  $\beta$ -catenin to activate target genes that have essential functions in the formation of the dorsal embryonic axis and in mesoderm development.

We previously investigated the role of *tcf1* as a downstream regulator of Wnt signaling in early gastrula stages by performing microarray analysis to identify genes differentially regulated upon *tcf1* overexpression (van den Broek and Destree, personal observations). Many genes expressed on the dorsal side of the embryo were found to be upregulated by overexpression of *tcf1*, including known direct  $\beta$ -catenin/TCF target genes such as *siamois* (*sia1*), *xnr3*, and *dkk1* (Brannon *et al.*, 1997; Chamorro *et al.*, 2005; McKendry *et al.*, 1997). Most of the downregulated genes are known to be expressed in the ventrolateral zone of the embryo, such as *cdx1* (*Xcad*) and *wnt8* (Christian *et al.*, 1991; Pillemer *et al.*, 1998). One of the identified upregulated genes was *foxd3*, which is an essential regulator of dorsal mesoderm development (Steiner *et al.*, 2006) and at later stages has a role in the formation of neural crest cells (Kos *et al.*, 2001; Lister *et al.*, 2006; Pohl and Knöchel, 2001; Sasai *et al.*, 2001). Furthermore, *Foxd3* has also been shown to be involved in regulating pluripotency in mouse embryonic stem cells (Liu and Labosky, 2008).

Because *foxd3* expression overlaps with that of *tcf1* at the dorsal blastopore lip during *Xenopus* gastrulation, we investigated whether *tcf1*/ $\beta$ -catenin complexes regulate

*foxd3* expression during mesoderm formation. By using morpholino injections, chromatin immunoprecipitation and other methods, we demonstrated that during gastrulation, endogenous *tcf1* is necessary and sufficient for activating the expression of *foxd3* and confirmed that this dorsal mesoderm regulating transcription factor is a Wnt/ $\beta$ -catenin target gene.

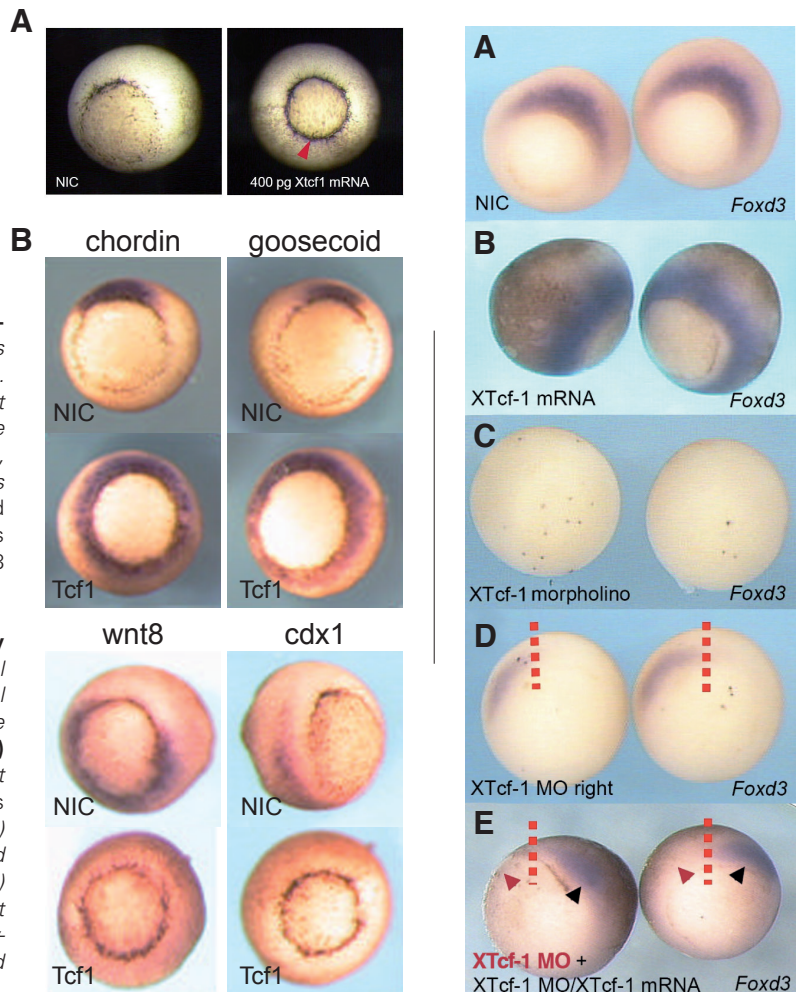
## Results

### Overexpression of *tcf1* dorsalizes *Xenopus* embryos and induces *foxd3*

To determine the function of *tcf1* in early gastrulation, *Xenopus tropicalis* embryos were injected with synthetic *Xtcf1* mRNA at the two-cell stage. When non-injected embryos had reached stage 10.5, these *tcf1*-overexpressing embryos displayed premature mesodermal involution around the entire marginal zone, consistent with a dorsalized phenotype (Fig. 1A). To confirm the dorsalized phenotype of the embryos overexpressing *tcf1*, we analyzed the expression patterns of the dorsal organizer genes *chordin* (Sasai *et al.*, 1994) and *goosecoid* (Cho *et al.*, 1991) and of the ventrolateral markers *wnt8* (Christian *et al.*, 1991) and *cdx1* (Pillemer *et al.*, 1998) by *in situ* hybridization (Fig. 1B). Upon *tcf1* overexpression, *chordin* and *goosecoid* were expressed all around the premature circular blastopore and expression of *wnt8* and *cdx1* became undetectable, indicating an expansion of organizer tissue at the expense

**Fig. 1 (left). Overexpression of *tcf1* induces a dorsalized phenotype in *Xenopus* embryos.** (A) *Xenopus tropicalis* embryos overexpressing *tcf1* display premature mesodermal involution. Stage 10.5 embryos, marginally injected in both blastomeres at the two-cell stage with 180 pg of *tcf1* RNA, show premature mesodermal involution around the entire marginal zone (81%,  $n=62$ ; red arrowhead, ventral). (B) Overexpression of *tcf1* induces dorsalization. Expression of *chordin* (83%,  $n=58$ ) and *goosecoid* (83%,  $n=59$ ) has expanded all around the blastopore in *Xenopus laevis* embryos injected with *tcf1* RNA, while expression of *wnt8* (73%,  $n=64$ ) and *cdx1* (89%,  $n=55$ ) is lost.

**Fig. 2 (right). *Tcf1* regulates the expression of *foxd3* in the early mesoderm.** (A) Endogenous expression of *foxd3* at the dorsal blastopore lip. (B) Injection of *tcf1* RNA in each blastomere (total of 400 pg) in two-cell stage *Xenopus laevis* embryos leads to the expression of *foxd3* all around the blastopore (75%,  $n=61$ ). (C) Injection of 10 ng of *tcf1* morpholino (MO) in both blastomeres at the two-cell stage abrogates *foxd3* expression in *Xenopus laevis* (50%,  $n=86$ ). (D) Unilateral injection of *tcf1* MO (right blastomere) at the two-cell stage inhibits *foxd3* expression only on the injected side (51%,  $n=84$ ). (E) Coinjection of *Xtltcf1* RNA (right blastomere) with *tcf1* MO (both blastomeres) rescues *foxd3* expression (right blastomere) in *Xenopus laevis*; left side is indicated by a red arrowhead, right side is indicated by a black arrowhead. (NIC, non-injected stage 10.5 control embryo).



of ventrolateral tissue. These results show that overexpression of *tcf1* during blastula and early gastrula stages dorsalizes *Xenopus* embryos, which is in line with the previously reported effects of *tcf1* overexpression in oocytes (Standley *et al.*, 2006).

One other gene affected by *tcf1* overexpression was *foxd3* (*XFD-6*). This transcription factor is implicated in mesoderm development and subsequent embryonic axis formation as well as in later neural crest development (Kos *et al.*, 2001; Lister *et al.*, 2006; Pohl and Knöchel, 2001; Sasai *et al.*, 2001; Steiner *et al.*, 2006). Steiner *et al.*, showed that *foxd3* is required as a transcriptional repressor at the *Xenopus* Spemann organizer to maintain expression of nodal-related genes essential for dorsal mesoderm formation (Steiner *et al.*, 2006). However, *foxd3* has not yet been identified as a downstream target of *tcf1*.

#### **Tcf1 is necessary and sufficient for regulating foxd3 expression**

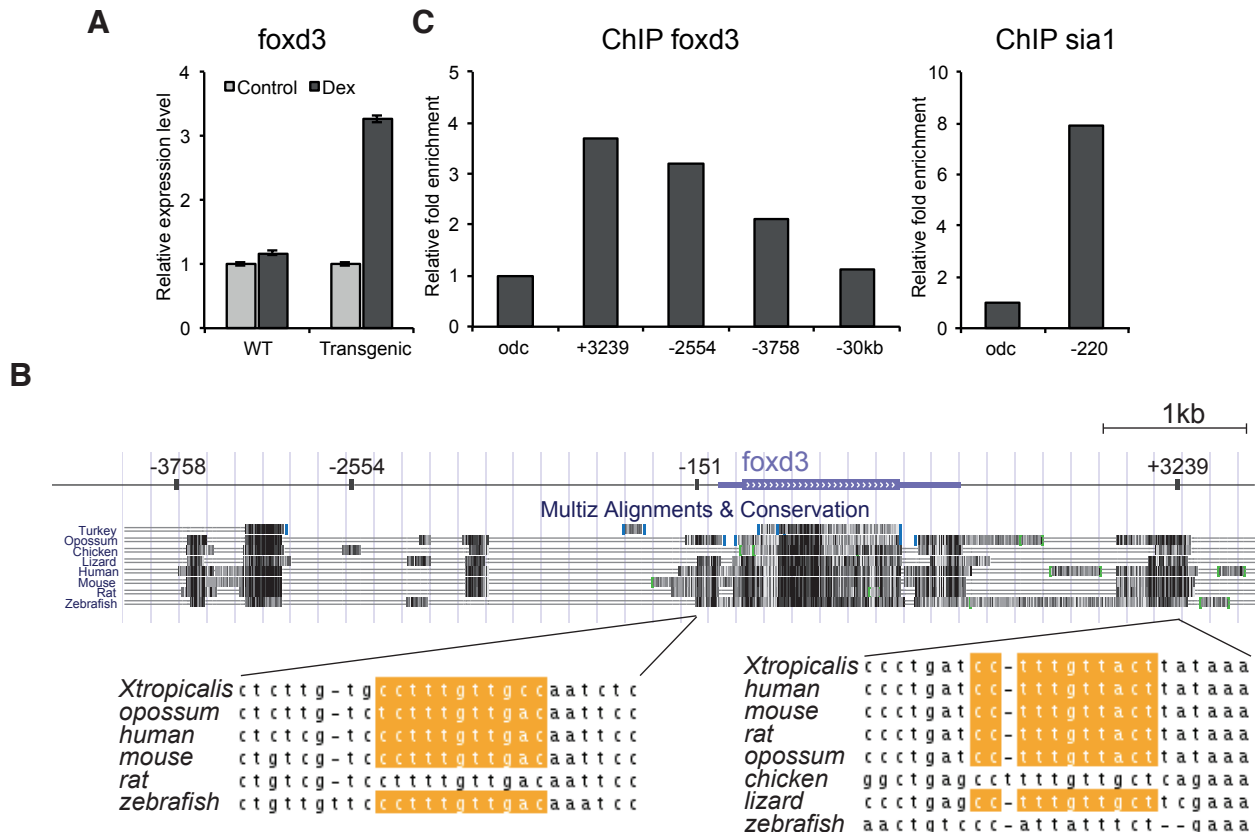
To confirm that *foxd3* is a target of *tcf1*, we manipulated *tcf1* expression in *Xenopus laevis* embryos and studied the effects on *foxd3* expression by *in situ* hybridization. At stage 10.5, endogenous expression of *foxd3* was highest in the dorsal lip (Fig. 2A), as reported before (Pohl and Knöchel, 2001; Steiner *et al.*, 2006). Overexpression of *tcf1* by injection of 400 pg of synthetic *tcf1* mRNA at the two-cell stage resulted in ectopic expansion of

*foxd3* expression all around the circular blastopore (Fig. 2B).

Conversely, inhibition of *tcf1* expression by injection of a translation blocking morpholino (MO) in both blastomeres at the two-cell stage abolished *foxd3* expression (Fig. 2C), indicating that early *foxd3* expression is dependent on endogenous *tcf1*. Furthermore, unilateral inhibition of *tcf1* expression resulted in loss of *foxd3* only on the injected side of the embryo (Fig. 2D), demonstrating that induction of *foxd3* by *tcf1* is likely cell-autonomous and that secreted inducing factors are not involved in the response to *tcf1*. The effect of *tcf1* morpholino (MO) could be rescued by co-injection of modified, MO-unsusceptible XtTcf1 mRNA (Liu *et al.*, 2005) (Fig. 2E, 44 of 80 affected embryos rescued), showing that the loss of *foxd3* is specifically related to the inhibition of *tcf1* expression. These experiments collectively reveal that transcription of *foxd3* in the earliest phases of its zygotic expression is dependent on *tcf1* at the dorsal lip of the blastopore.

#### **Foxd3 is controlled by Wnt/ $\beta$ -catenin signaling during gastrulation**

In early *Xenopus* embryos, *tcf1* is strongly expressed in almost the entire marginal zone, except for a narrow region around the blastopore (Roël *et al.*, 2003) and this pattern overlaps with the presence of nuclear  $\beta$ -catenin during gastrulation (Schohl and Fagotto,



**Fig. 3. Quantitative RT-PCR and chromatin immunoprecipitation (ChIP) indicate that *foxd3* is a direct target of Wnt/ $\beta$ -catenin signaling during gastrulation.** (A) *Foxd3* is induced upon artificial Wnt activation during gastrulation. *Xenopus tropicalis* wild type embryos (WT) and embryos transgenic for a Dexamethasone (Dex) inducible Wnt activating transgene were subjected to Dex or control treatment for 2.5 h during gastrulation. (B) Schematic overview of LEF/TCF consensus sites at the *X. tropicalis foxd3* gene locus. Evolutionary conservation around the position -151 and +3239 relative to the transcription initiation site is shown. (C) ChIP assay on WT *X. tropicalis* gastrulating embryos detects binding of  $\beta$ catenin/lef/tcf complexes to the *foxd3* locus. *Sia1* was included as a positive control and a region in the *odc* gene served as a negative control. Data are shown as fold enrichment relative to the negative control.



2002). Consequently, we tested whether *foxd3* is directly regulated by endogenous Wnt/ $\beta$ -catenin signaling during gastrulation.

First, we investigated if *foxd3* expression is under the control of Wnt/ $\beta$ -catenin signaling during mesoderm formation. We used transgenic *X. tropicalis* embryos that contain an optimized hormone-inducible system allowing activation of the Wnt pathway at the transcriptional endpoint (Denayer et al., 2008). The activator part of this transgenic system consists of a chimeric fusion of the LEF/TCF DNA binding HMG box of murine LEF1, fused to the transactivation domain of the *Herpes simplex* virus VP16 gene. To make this transgene inducible, the hormone binding domain of the human glucocorticoid receptor was added, resulting in a dexamethasone (Dex) inducible system for activating LEF/TCF target genes.

Transgenic and wild type embryos were obtained by natural mating and induced during gastrulation by the addition of 10  $\mu$ M Dex to the cultivation buffer for 2.5 h. Afterwards, embryos were lysed, RNA was extracted and quantitative RT-PCR was performed to analyze *foxd3* expression. As Fig. 3A shows, when artificial Wnt/ $\beta$ -catenin was activated, *foxd3* mRNA was upregulated three-fold.

To investigate whether regulation of *foxd3* by *tcf1* is a direct effect of the binding of  $\beta$ -catenin/*tcf1* to *foxd3* regulatory sequences, we first performed an *in silico* screening of the *foxd3* locus of *X. tropicalis* for the presence of potential *tcf1* binding sites. This uncovered five regions in the *foxd3* locus, at positions +3239, -151, -2554 and -3758 and one site more upstream of the *foxd3* transcription start site, at -30 kb (Fig. 3B).

To determine whether *tcf1* is associated with chromatin at these conserved TCF binding sites in the *foxd3* locus, we wanted to perform chromatin immunoprecipitation (ChIP). Unfortunately, antibodies recognizing *Xenopus* *tcf1* usable for ChIP assays are missing. However,  $\beta$ -catenin is the nuclear interaction partner of LEF1/TCF factors necessary for transmitting Wnt/ $\beta$ -catenin signaling. Hence, we performed ChIP assays on cross-linked chromatin of gastrula stage *X. tropicalis* embryos with a polyclonal antibody against  $\beta$ -catenin. A  $\beta$ -catenin/ChIP assay for the known Wnt target gene *siamois* (*sia1*) was included as a positive control (Brannon et al., 1997). Immunoprecipitated DNA was then used for qPCR with primers that flank the putative *lef1/tcf* binding sites. To correct for the background signal, we normalized the data against DNA immunoprecipitated with rabbit IgG. Several independent experiments revealed enrichment in the *foxd3* locus at positions +3239, -2554 and -3758. The region located farther upstream at 30 kb was not immunoprecipitated (Fig. 3C). Unfortunately, the predicted *lef1/tcf* binding site at position -151 is located in a highly conserved region that is resistant to PCR amplification, as reported before by Nelms et al., (Nelms and Labosky, 2011). Consequently, the binding of  $\beta$ -catenin/*tcf* complexes to this site could not be evaluated. Hence, we could confirm binding of *tcf1*/ $\beta$ -catenin complexes at least at three *lef1/tcf* binding sites surrounding the *foxd3* locus.

## Discussion

### *Tcf1* is required for expression of *foxd3*

We found that *tcf1* overexpression is correlated with the induction of dorsal mesoderm and with the ectopic expression of *foxd3* during *Xenopus* gastrulation. Previous *tcf1* loss-of-function experiments have documented its role in the induction of mesodermal markers (Liu et al., 2005), and Pohl et al., reported that *foxd3* can be ectopi-

cally activated on the ventral side of the gastrulating embryo by experimentally activated Wnt signaling (Pohl and Knöchel, 2001). Consistent with the overlap of *tcf1* expression (Pohl and Knöchel, 2001; Roël et al., 2003; Steiner et al., 2006) with the earliest zygotic expression of *foxd3* (i.e., in the dorsal blastopore lip), we could confirm that *foxd3* is directly regulated by Wnt/ $\beta$ -catenin signaling in the dorsal mesoderm. Using  $\beta$ -catenin ChIP experiments, we identified three Wnt responsive *lef1/tcf* binding sequences at the *foxd3* gene locus. Of these, the +3239 site, as well as its surrounding sequences, shows high evolutionary conservation (Fig. 3B). The other two sites are positioned in regions of the *foxd3* promoter that show virtually no evolutionary conservation. Notice that the PCR-resistant -151 *lef1/tcf* site is also highly conserved (Fig. 3B). ChIP enrichment of the *foxd3* locus was not as strong as that of the *sia1* promoter, which served as a positive control. Nonetheless, we could confirm enrichment at the *foxd3* locus in every  $\beta$ -catenin ChIP experiment we performed. Furthermore, it is possible that  $\beta$ -catenin interacts predominantly at the PCR-resistant -151 site, or that the high GC content and consequent secondary structures that form at the *foxd3* promoter (Nelms and Labosky, 2011) can interfere with the ChIP assay.

There are no ChIP compatible antibodies described that cross-react with *Xenopus* *lef1/tcf* factors. Consequently, we could not identify whether endogenous *tcf1*, *lef1*, *tcf3* or *tcf4* binds to these sites. Nevertheless, *tcf3* acts as a transcriptional repressor of organizer genes (Brannon et al., 1999; Houston et al., 2002), *tcf4* transcripts are present at levels below the detection limit of *in situ* hybridization (König et al., 2000; Standley et al., 2006), and *lef1* is primarily expressed at the ventrolateral marginal zone (Roël et al., 2009). On the basis of these findings and on the enhancement of *foxd3* expression by *tcf1* overexpression, we conclude that *tcf1* is probably the *lef1/tcf* factor responsible for activation of *foxd3* in the Spemann organizer.

### *Tcf1* regulates *foxd3* in dorsal mesoderm formation

Cooperative Wnt and FGF signaling is crucial for dorsal mesoderm development (Christian et al., 1992; Keenan et al., 2006). Also, *foxd3*, a transcriptional repressor required for dorsal mesoderm formation (Steiner et al., 2006), is downregulated when FGF signaling is blocked during gastrulation (Branney et al., 2009). These results, complemented with our data, indicate that regulation of the endogenous dorsal expression of *foxd3* relies on combined activation through Wnt and FGF dependent mechanisms. A recent model proposes that FGF signaling can stimulate Wnt-mediated mesodermal gene activation via MAPK phosphorylation of the groucho/TLE protein groucho-related 4 (*grg4*), relieving its repressive interaction with *lef1/tcf* proteins (Burks et al., 2009). In addition, *grg4* is co-expressed with *tcf1* and enriched at the dorsal mesoderm during gastrulation (Molenaar et al., 2000; Roël et al., 2003). Based on this mechanism and the observation that *foxd3* can be positively regulated by FGF signaling during *Xenopus* gastrulation (Branney et al., 2009), it is possible that *foxd3* expression is regulated through a balance between *tcf1*/ $\beta$ -catenin activating complexes and *tcf1*/groucho repressing complexes, the latter of which can be modulated by MAPK phosphorylation via FGF signaling.

Interestingly, *foxd3* can bind to *grg4*. This recruitment of *grg4* seems to enhance the transcriptional repressor function of *foxd3*, which is essential for dorsal mesoderm development. This sug-

TABLE 1

## SEQUENCES OF PRIMERS USED FOR qRT-PCR AND ChIP

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>foxd3</i> (cDNA)	GAGGACATGTTGACAATGG	CAAAGCTTTGCATCATGAGAG
<i>tubb</i> (cDNA)	GACCCCACTGGCACCTATCA	TCGAGGGACATATTTACCACCTGT
<i>odc</i> (cDNA)	TTTGGTGCCACCCTTAAACA	GCCACTGCCAACATGGAAC
<i>foxd3</i> (+3239)	CTGATCCTGTGCTTCTAAATGAC	TGCTATGTGCAGAAATATTCGG
<i>foxd3</i> (-2554)	CCAACTGAAGGCTCCATCAAG	AAAGGGGAATCAAAGGTGTCCT
<i>foxd3</i> (-3758)	TGGGAAGAGCACAAAGTGTGAT	TTACAGCCAGAGCGATTA
<i>foxd3</i> (-30kb)	TGTGGAAGTCAAAGGAGAAAAA	GGAACAGCGTTCAATAGCTTGT
<i>sia1</i> (-220)	AAGATCAAGGGAACAGGTG	TTGCACCTACAAACATGGG
<i>odc</i> genomic	GTGCACGCCTGAATCTTCT	GGCTCAGCAATGATGGTCACT

gests potential re-use of *grg4* by *foxd3* after its own activation through Wnt signaling.

In conclusion, our results show that *foxd3* is a direct target of *tcf1* during *Xenopus* gastrulation and that Wnt/ $\beta$ -catenin mediated activation of *foxd3* via *tcf1* is essential for dorsal mesoderm induction and axis formation during early embryonic development.

## Materials and Methods

### In situ hybridization

Whole-Mount RNA *in situ* hybridization was performed as described in Molenaar *et al.*, (Molenaar *et al.*, 1998), except that hybridization was carried out at 65°C. Digoxigenin-labeled antisense probes were synthesized with the Ambion Maxiscript kit and digoxigenin-UTP from Roche. The digoxigenin-labelled antisense RNA probes used were *Xenopus* chordin (Sasai *et al.*, 1994), *Xenopus* goosecoid (Cho *et al.*, 1991), *Xenopus* wnt8 (Christian *et al.*, 1991), *Xenopus* cdx1 (Pillemer *et al.*, 1998) and *Xenopus* foxd3 (Dirksen and Jamrich, 1995). All experiments were repeated at least once.

### Morpholino and RNA injections

Capped *Xtcf1* (*tcf1*) mRNA was synthesized with the Ambion mMessage mMachine kit after *Xba*I linearization of the plasmid described in Liu *et al.*, (Liu *et al.*, 2005). *Xenopus laevis* embryos were injected with 400 pg of *Xtcf1* mRNA in both cells at the two-cell stage in the marginal zone. *Xenopus tropicalis* embryos were injected with a dose of 180 pg of *Xtcf1* mRNA. The *tcf1* morpholino and modified *tcf1* rescue construct have been described in Liu *et al.*, (Liu *et al.*, 2005). Morpholino was injected in *Xenopus laevis* at the two-cell stage into the marginal zone at 10 ng per blastomere. Embryos were fixed in MEMPHIA (100 mM MOPS, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 4% paraformaldehyde) and dehydrated in methanol.

### Chromatin immunoprecipitation

Consensus *lef1/tcf* binding sites were determined and evaluated for conservation by using the UCSC Genome Browser (genome.ucsc.edu) (Kent *et al.*, 2002), the Match tool of TRANSFAC (Biobase) (Kel *et al.*, 2003) and the alignment tool Contra (Hooghe *et al.*, 2008). ChIP assays were performed according to the method described before (Blythe *et al.*, 2009) but with minor modifications: we used gastrula *X. tropicalis* embryos at stage 11 and cross-linking time was 45 min. After homogenization, embryos were sonicated with a Brandson cell disruptor to obtain DNA fragments smaller than 1000 bp. Immunoprecipitation was performed with rabbit anti- $\beta$ -catenin polyclonal antiserum (a kind gift from Dr. Barry Gumbiner (McCrea *et al.*, 1993)). Purified normal rabbit IgG was purchased from Santa Cruz (SC-2027). Instead of sepharose beads, protein G Dynabeads (Invitrogen) were used.

### Real-time quantitative RT-PCR analysis

Total RNA was isolated using the Aurum Total RNA fatty and fibrous tissue kit (Biorad). For each RNA sample, at least 20 embryos were pooled. cDNA was prepared with oligo (dT) and random hexamer primers using the iScript

cDNA Synthesis Kit (Biorad) according to the manufacturer's instructions.

Real-time qPCR analysis was performed by using the SYBR green PCR master mix (Roche) on a LightCycler® 480 Real-Time PCR System (Roche). Primers for measuring gene expression levels (as well as for ChIP experiments) were designed by using Primer express 1.0 software (Perkin-Elmer applied Biosystems). The primer sequences are listed in Table 1. A "no-template control" was included for all primer pairs. When measuring gene expression, a "no amplification control" was included and all values were normalized to the level of the housekeeping genes ornithine decarboxylase (*odc*) and tubulin beta (*tubb*).

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